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Investigation of terahertz radiation influence on rat glial cells

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Abstract: We studied an influence of continuous terahertz (THz) radiation (0.12 - 0.18 THz, average power density of 3.2 mW/cm^2) on a rat glial cell line. A dose-dependent cytotoxic effect of THz radiation is demonstrated. After 1 minute of THz radiation exposure a relative number of apoptotic cells increased in 1.5 times, after 3 minutes it doubled. This result confirms the concept of biological hazard of intense THz radiation. Diagnostic applications of THz radiation can be restricted by the radiation power density and exposure time.

OCIS codes: (170.0170) Medical optics and biotechnology; (170.3660) Light propagation in tissues.

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1. Introduction

Over the last several decades, terahertz (THz) technologies have been developing quite rapidly. Nowadays, THz radiation (0.1-10 THz) is applied in a variety of scopes, including medicine [1]. For the effective development of diagnostic and therapeutic techniques, it is essential to be aware of fundamental biological effects of THz radiation. It is known that THz radiation causes a

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variety of biological effects, including some at the cellular level. Detailed reviews on this issue can be found in papers [2–7]. Influence of THz radiation on cells is revealed in the change of genes activity and cell membrane status. It was also reported before, that THz radiation changes the electrical charge of the membrane of human red blood cells [8, 9], causes a violation of the adhesive properties of the nerve cell membrane of a snail and mollusk [10, 11]. An indication of the structural damage is the increase in membrane permeability, as it was shown in some experiments with human red blood cells and lymphocytes [12], and laboratory rats red blood cells [13, 14].

A major part in the cells response to the THz radiation is determined by the intercellular influence. It is acknowledged that monocytes are regulators of lymphocyte activity. The regulation is carried out by cytokines, special signaling substances. It has been delineated that co-incubation of lymphocytes and monocytes after simultaneous or separate exposure, incubation of irradiated lymphocytes with non-irradiated monocytes and incubation of irradiated monocytes with non-irradiated lymphocytes have different effects on lymphocyte proliferative index. The index has the highest value in case of the co-incubation of monocytes and lymphocytes after separate exposure and has the lowest value in case of the incubation of irradiated lymphocytes with non-irradiated monocytes [12].

According to the authors, these differences are caused by the monocytes status as irradiated monocytes influence even non-irradiated lymphocytes and increase their proliferative index [12]. Monocytes also affect the viability of cells. Thus, in case of simultaneous exposure of lymphocytes and monocytes derived from the same blood sample, the percentage of viable lymphocytes decreases in comparison with that in a control sample, whereas in cases of separate irradiation or incubation of irradiated lymphocytes with non-irradiated monocytes, this indicator increases. In event of incubation of irradiated monocytes with non-irradiated lymphocytes, the percentage of unstained lymphocytes decreases [12].

All of the above experiments were carried out using preparations of isolated cells. Investigation of the THz radiation effect on the membrane permeability of a cell in a culture is of particular interest, considering the fact that the properties of a single cell in a culture are defined by a number of factors caused by vital activity of neighboring cells and the intercellular relationships. In addition, the condition of the cells in a culture depends on the parameters related to the condition of a culture in general. In particular, a significant contribution is made by the regulation of cellular status using chalones [15]. Such a multifaceted effect will determine the nature of the response of cells in a culture to the THz radiation.

In particular, a heterogeneous response in the experiments with cultures of kangaroo rat kidney cells (cell line PTK2) [16] and Chinese hamster ovary [17] were demonstrated. A part of the cells revealed a decrease in the number of 3H-thymidine incorporation (DNA synthesis precursor), which is considered by the authors as an inhibition of DNA synthesis. The largest effect was observed in the cells being in S-phase of the cell cycle during exposure. Furthermore, it was shown that the THz exposure to the inclusion of labeled uridine (RNA synthesis precursor) had no effect. This difference may be a result of the THz radiation effect on the cell membrane condition.

Among the cellular effects, the cytotoxic effect of THz radiation is also an issue of concern for many scientists. Currently, there is no consensus on this issue. Some studies have demonstrated that this effect does not occur [18–25], whereas in other works the effect was clearly displayed [10–12, 26–28]. Particularly, experiments in papers [19, 20, 25, 28] showed different results after exposure of THz radiation of approximately same frequency (0.10 - 0.15 THz) and power density (0.04 - 5 mW/cm^2). In all mentioned experiments, registration of the effect was carried out using adequate methods; however, the samples under exposure were different. It is possible that presence or lack of the effect is associated with the properties and characteristics of some particular cells. For this investigation, glial cells were selected as the samples of the experiment. They are highly sensitive to the ionic changes in environment [29]. One of the mechanisms

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of THz radiation impact on living systems may be a disturbing effect on the status of the cell endogenous field, which will lead to changes in the ionic fluxes from a cell to environment and vice versa, and affect cell viability.

2. Samples and methods

2.1. Cells

The investigation included experiments using C6 rat glial cell line, obtained from the Collection of cell cultures of vertebrates (Institute of Cytology of the Russian Academy of Sciences). The cells culturing was carried out in accordance with the recommendations of the Institute. For setting up the experiments, the cells were seeded into the wells of 24-well flat-bottom plate (Sarstedt, Germany). In the experiment, 20 wells of a plate were exposed, whereas samples in 4 wells were used as control ones. The cell monolayer with density of 50% was irradiated. After irradiation, the cells were incubated at 37°C in an atmosphere of 5% CO_2 during 24 hours. At the end of incubation, the wells were washed twice with an excess of Versene solution heated to 37°C (Biolot, St. Petersburg). For the monolayer disintegration, the Accutase solution (Sigma-Aldrich, USA) heated to 37°C was used; the incubation time was 7 minutes at 37°C under 5% CO_2. Upon completion of incubation, the cooled phosphate buffered saline (PBS, pH 7.2 - 7.4) containing 2% fetal calf serum (FCS) (Biolot, St. Petersburg, Russia) was added to the wells in order to inactivate Accutase. The resulting cell suspension was transferred to 15 ml centrifuge tubes (Sarstedt, Germany) and washed twice with PBS (300 g for 8 minutes). The resulting cell suspension was used for the experiments described below.

2.2. Assessment of changes in mitochondrial membrane potential

This method is based on the use of two fluorescent dyes: tetramethylrhodamine methyl ester (TMRM) and DNA-binding DRAQ7 dye [30]. TMRM corresponds to the group of cationic lipophilic dyes used for studying the mitochondrial membrane potential of cells. TMRM is able to freely penetrate through bilipid cell membranes and to accumulate in areas with high concentrations of protons, i.e., at the inner mitochondrial membrane, due to its cationic properties. This effect is accompanied by a change in the cells fluorescence intensity in the red-orange part of the spectrum (the maximum emission wavelength of 573 nm), which is recorded during the analysis by the flow cytometer. During the initial stages of the physiological cell death (apoptosis), the mitochondrial membrane depolarization occurs, and the proton concentration decreases, resulting in dye release in the cytoplasm and significant reduction of its fluorescence. Thus it is possible to distinguish the live cells with effectively functioning mitochondria (and, as a consequence, with high intensity fluorescence (phenotype “TMRM high"), from dying or dead cells in which mitochondrial functioning in disturbed (phenotype “TMRM low").

While mitochondrial depolarization is considered an “early" event at the apoptotic start, the violation of the membrane surface integrity (i.e., its fragmentation) is usually characteristic for the cells located at the terminal stages of death. Therefore, for identifying the various stages of apoptosis, besides TMRM, cells are further dyed by DRAQ7 - a dye capable of interacting with nucleic acids cells. Though DRAQ7 is not able to diffuse through the bilipid membranes and, consequently, to communicate with the cellular DNA, during the cytoplasmic and nuclear membranes fragmentation the dye enters the cell and interacts with DNA and RNA. The consequence of such interaction is the accumulation of the dye in the cytoplasm and nucleus and the fluorescence of the cell in the red part of the spectrum (detection is performed at a wavelength of 725 ± 20 nm).

Figure 1 shows gating tactics used for isolation of live cells and cells at different stages of apoptosis. Living cells have a bright fluorescence channel, intended to detect TMRM, but do not accumulate DRAQ7 - phenotype "TMRM high / DRAQ7 -". During the early stages of apoptosis,

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the cells have reduced mitochondrial potential, but retain tightness for DRAQ7 - phenotype "TMRM low / DRAQ7 -" .The cells at a late stage of apoptosis or already dead (necrosis) do not effectively accumulate TMRM, but are dyed by DRAQ7 - phenotype "TMRM low / DRAQ7 +".

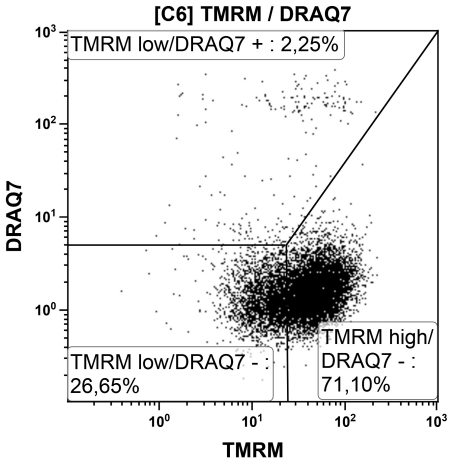

Fig. 1. Gating tactics used for isolation of live cells and cells at different stages of apoptosis.

2.3. Cell labeling procedure

For the assessment of the mitochondrial membrane potential, a tetramethylrhodamine methyl ester (TMRM (Cat. # T668, Thermo Fisher Scientific Inc, USA)) solution was added to 100 μl of cell suspension. The final TMRM concentration was 150 nM. Samples were thoroughly mixed up and incubated for 20 minutes at 37°C under 5% CO_2 atmosphere. Upon the incubation completion,the samples were washed with excess PBS containing 2% FCS (8 minutes at 300 g). Then the supernatant was removed and the cell pellet was resuspended into 100 μl of fresh PBS. The 5 μl of the working solution of the DNA binding dye DRAQ7 (Cat. # B25595, Beckman Coulter, USA) was added to the resulting cell suspension, and samples were incubated for 10 minutes at room temperature, protected from light. At the end of incubation, the 200 μl of PBS was added to the samples and the cytometric account was conducted.

2.4. Flow cytometry

Flow cytometry analysis of the samples was performed on a Navios flow cytometer (Beckman Coulter, USA). For each of the samples at least 20,000 single cells were analyzed. To distinguish single cells from sticking together and to discriminate the aggregates from the analysis, the following combinations of the signals on the forward (a quantity proportional to the size of the cells, FS) and side (quantity characterizing the structure of cells, SS) light scattering was used: the intensity of the peak against the intensity of the integrated FS or SS signals as well as the time of the cell flight (TOF) through the detection zone against the integral FS or SS signals intensity. Analysis of the results was performed using Kaluza 1.3 software (Beckman Coulter, USA). The data was processed using Microsoft Office Excel (Microsoft, USA), Statistica 8 (StatSoft, USA) and GraphPad Prism (GraphPad Software, USA) software and applications packages. The result is reported as an arithmetic mean with an error as the standard error of mean (Mean ± SEM). Comparison of samples was performed using Student's t-test. Differences were considered reliable at $p < 0.05$.

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2.5. Experimental setup

In order to irradiate cell culture with broadband THz radiation, a backward wave oscillator (BWO) source operating in 0.12 - 0.18 THz frequency range was used [31]. The experimental setup is shown in the Fig. 2.

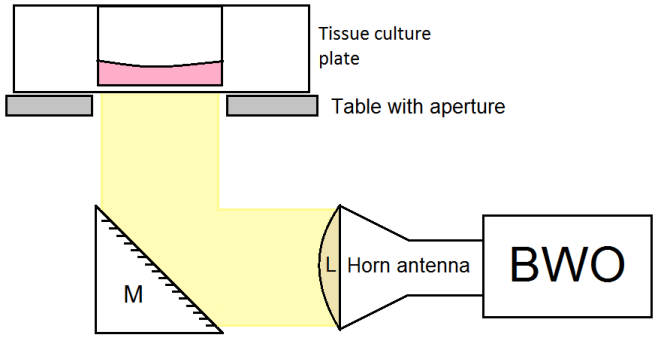

Fig. 2. Experimental setup: THz radiation emitted by BWO is fed into horn antenna and collimated using TPX lens to obtain 20 mm wide beam. The beam is directed by the mirror to propagate orthogonally to the bottom surface of Petri dish filled with cell culture being irradiated.

The BWO emitted radiation of approximately 10 MHz frequency span at central frequency of 0.15 THz with average power of 10 mW and average power density of 3.2 mW/cm^2. The output of the BWO was terminated with a horn antenna with mounted planoconvex TPX lens in order to get 20 mm wide collimated beam (Fig. 2). Mirror with thickness of gold coating sufficient to reflect incident THz radiation was used to direct the beam orthogonally to the bottom surface of the transparent tissue culture plate. The series of experiments were performed under room temperature, the time duration of THz radiation exposure was 1, 2, 3, 4, and 5 minutes.

3. Results

For analysis of the results two-dimensional fluorescence intensity histogram of TMRM (the fluorescence increase depends on the membrane potential of mitochondria) and Draq7 (penetrating into the cells during the late stages of apoptosis and necrosis after destroying the surface membrane integrity) were constructed. The obtained data is shown in the Table 1 and Fig. 3. It is demonstrated that the number of cells with active functioning mitochondria (phenotype "TMRM high / Draq7-") in the samples exposed to THz radiation is significantly lower than the same characteristic in the control samples ($p < 0.05$). Increasing the exposure time leads to the decrease of the amount of such cells in the sample. In turn, the number of cells with a reduced ability to accumulate mitochondrial dye (phenotype "TMRM low"), significantly increases with the exposure time. It should be mentioned that in a population of phenotype "TMRM low / Draq7 +" cells, i.e. cells with decreased mitochondrial membrane potential and an outer membrane damage, no significant change in the test samples compared to the control samples was noticed.

It should be mentioned, that the effects observed after the THz radiation exposure were not thermal: during the exposure, the temperature change did not exceed 0.1°C [32].

4. Discussion

According to the literature analysis, THz radiation has no cytotoxic effect while interaction with some types of cells, but causes this effect while interaction with other cells types under similar

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Table 1. Relative number (in %) of live cells and cells at different stages of apoptosis in samples irradiated by THz emission of different durations (Mean ± SEM, n = 4, *p < 0.05, **p ⩽ 0.001)

	Live cells	Early apoptotic cells	Late apoptotic/necrotic cells
exposure	Live cells (TMRM high/Draq7-)	Early apoptotic cells (TMRM low/Draq7-)	Late apoptotic/necrotic cells (TMRM low/Draq7+)
0 min	88.35 ± 1.2	9.98 ± 1.23	1.68 ± 0.24
1 min	82.18 ± 1.4*	15.6 ± 1.12*	2.23 ± 0.59
2 min	81.9 ± 0.46*	15.83 ± 0.49*	2.28 ± 0.63
3 min	79.65 ± 0.98**	18.93 ± 0.97**	1.43 ± 0.07
4 min	76.5 ± 1.6**	21.75 ± 1.55**	1.75 ± 0.14
5 min	74.3 ± 1.73**	24.28 ± 1.68**	1.43 ± 0.11

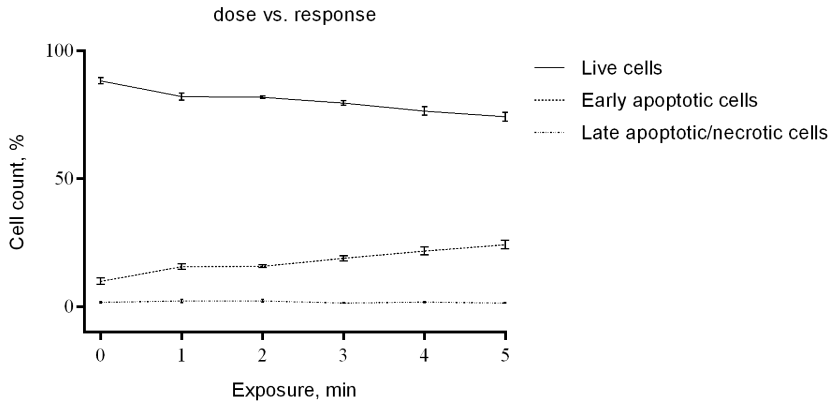

Fig. 3. The number of live cells and cells at early and late stages of apoptosis in the sample in relation of the THz radiation exposure time.

conditions of exposure. It is noteworthy that a lack of cytotoxic effect happens if covering and supporting tissue cells (epithelial cells [19, 33], fibroblasts [20] and keratinocytes [22, 23]) are investigated. We suggest that a lack of sensitivity has a function of shielding a body from the THz radiation. The second type of cells indifferent to THz radiation is stem cells [21, 24]. It can be assumed that in this case a membrane also functions as a screen which does not allow any impact of the external THz field on the process of differentiation. The third type of non-sensitive cells is bacterial cells [18]. We believe that in this case a membrane also protects a microorganism from the external THz field.

The cytotoxic effect is typical for lymphocytes [12, 26–28], neurons [10] and red blood cells [12], i.e. to cells located within a body. It is possible that local THz nano- and microfields essential for an organism functioning are generated within it. There are some physical prerequisites for this theory. Due to all mentioned reasons, all cells located within a body must be highly sensitive to THz radiation. Possible mechanisms of THz radiation influence are described below. Since many of the intracellular structures of glial cells are significantly polarized [29], and the size of glial cells is less than a wavelength of the used THz source, a direct, up to a resonance, absorption of radiation by glial cells is a possible mechanism.

Moreover, the culture of glial cells is located in an aqueous medium. Water has very high absorption of THz radiation [34]. The energy absorbed by water can trigger a change of state of

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membrane proteins molecules. In addition, by changing the water condition, THz radiation may have an impact on those biochemical processes that can be modified to some extent by the change of the water molecules structure, solvation shell of molecules or system of hydrogen bonds.

Furthermore, glial cells have a high membrane potential. It is higher than that of neurons. This helps to maintain intracellular homeostasis and ion exchange between neurons and glial cells. In addition, glial cells are characterized by high density of proteins associated with ion channels at the cell surface membrane [29]. The impact of THz radiation of a wavelength larger than glial cell sizes is disturbing. This leads to depolarization of a cell membrane, and membranes of lysosomes and mitochondria. This is confirmed by registered appearance of TMRM dye in the cytoplasm under exposure. The result of membranes depolarization is the change of membrane proteins conformation, which makes it impossible to restore the intracellular homeostasis and leads to the release of apoptogenic proteins from the mitochondria into the cytoplasm, and release of proteolytic enzymes from lysosomes with the further development of the apoptosis process.

Another feature of glial cells is the presence of a large amount of aquaporin, the channel protein [35]. Since THz radiation induces conformational transitions in protein molecules, it was reasonable to expect a change of aquaporins conformation and, consequently, enhanced water inflow into the cell, and cell membrane deformation due to cell swelling. However, the relative number of cells with defragmented cell membrane was virtually unchanged throughout the experiment. This is evidenced by the absence of DRAQ7 dye accumulation. This does not mean that such a mechanism of the cells destruction is excluded. But for its pronounced manifestation other experimental conditions are required.

The results obtained in this study are additional evidence that THz radiation can pose a biological hazard. This issue was already raised in scientific literature [1, 3, 28, 36].

5. Conclusion

In this investigation, we demonstrated a dose-dependent cytotoxic effect of THz radiation on rat glial cells. In the experiment, a C6 rat glial cell line was exposed by continuous THz radiation (0.12 - 0.18 THz) at average power density of 3.2 mW/cm^2. After one minute of exposure, a relative number of apoptotic cells increased by a factor of 1.5, after 5 minutes it became 2.4 times higher than the initial value. This result confirms the concept of biological hazard of intense THz radiation. Therefore, we claim that diagnostic applications of THz radiation can be restricted by the radiation power density and exposure time.

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This paper is dedicated to the blessed memory of Prof. Viacheslav Fedorov, who passed away on September 27th, 2016.